Nodulation: **Finding the lost common denominator**

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The products of the 'common' nodulation genes of *Rhizobium* catalyze the synthesis of signal molecules and were once thought to have similar functions in all *Rhizobium* species; subtle differences in the activities of these gene products have now been discovered that influence the host range of *Rhizobium* species.

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Bacteria in the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively known as rhizobia, form complex symbiotic relationships with legume roots in the form of nitrogen-fixing nodules (Fig. 1). This association is established in a host-specific and guest-specific manner that involves a two-way exchange of chemical signals. The first signal comes from the host plant, in the form of a mixture of flavonoids released from the legume root. This signal induces the synthesis by receptive nodulating bacteria of signal molecules known as nod factors. Nod factors cause the legume root cells to become mitotically active and to differentiate into nodule structures. These factors are composed of chitin-oligosaccharide backbones modified with a variety of substituents (reviewed in [1]), and are synthesized by enzymes encoded by the bacterial nodulation genes.

The nodulation genes have been classified as either 'common' or 'host-specific'. A nodulation gene is considered common if it can be complemented by a gene from another species without altering the host-range of the recipient bacterium. For example, *Rhizobium meliloti* forms nodules with the host plant alfalfa, but not with vetch, and the converse is true of *Rhizobium leguminosarum* biovar *viciae*. *R. meliloti* with a mutation of a common nodulation gene can be complemented to form nodules on alfalfa by cloned DNA from *R. leguminosarum* bv. *viciae*. These *R. meliloti* transconjugants cannot nodulate vetch, so their host range was unchanged [2]. This same theme has been found across quite diverse species of *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* [3–6]. Host-specific nodulation genes, on the other hand, cannot functionally complement mutations across species and often alter the host range of the recipient rhizobia when transferred from one species to another.

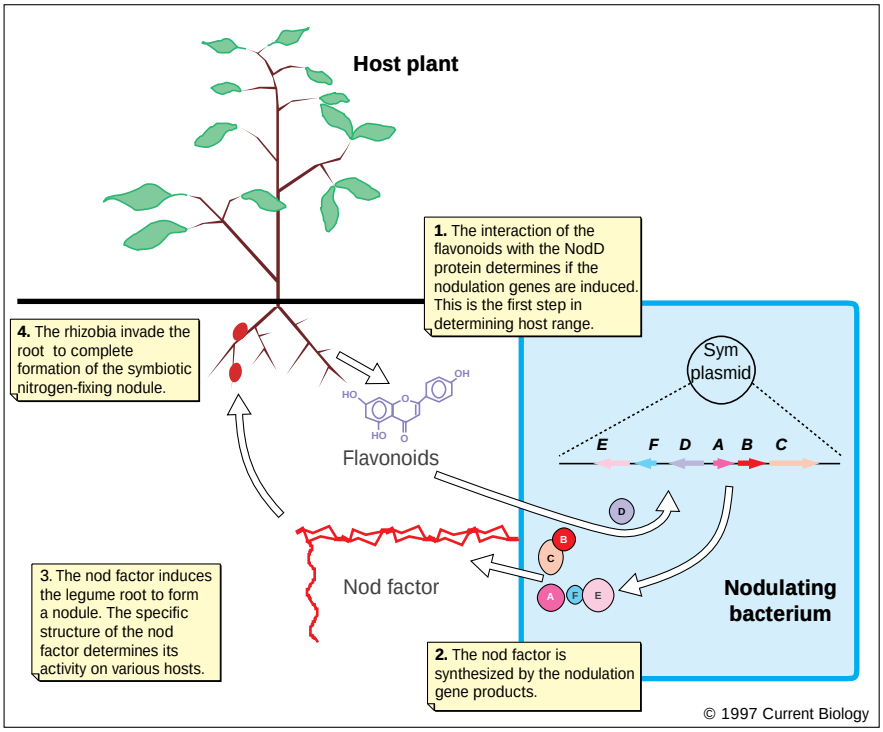
The *nodD* and *nodABC* genes were originally defined as common nodulation genes and their homologues were thought to have similar functions in the various different species of rhizobia [2]. Support for this view came from DNA sequence data for the common nodulation genes, revealing strong conservation among rhizobia species. However, as more is learnt about the enzymatic functions of the products of the common and host-specific nodulation genes, this hypothesis is becoming less tenable — it is now clear that some nodulation genes that were initially considered to be 'common' actually encode products that exhibit an element of host specificity.

The common nodulation genes encode enzymes that catalyse the synthesis of the nod factor core (Fig. 2). Specifically, the NodC protein is a chitin-oligosaccharide synthase that polymerizes UDP–*N*-acetyl-D-glucosamine into chitin-tetraose and chitin-pentaose [7], and NodB protein deacetylates the terminal *N*-acetylglucosamine at the non-reducing end of these chitin-oligosaccharides [8,9]. The NodA protein is an acyltransferase that adds an *N*-linked acyl substituent to the deacetylated chitin-oligosaccharide [9]. The identity of the *N*-linked acyl moiety is determined by the host-specific genes *nodEF*, the products of which assist in the synthesis of special, polyunsaturated fatty acids.

The addition of special fatty acids is required for optimal activity of the nod factors on their respective legume hosts. The nod factors made by both *R. meliloti* [10] and *R. leguminosarum* bv. *viciae* [11] have acyl substituents, though they are different in the two cases. Thus *R. meliloti* and *R. leguminosarum* have homologous *nodEF* genes encoding enzymes that have similar activities, but that assist in the synthesis of different, polyunsaturated fatty acids. In contrast, some species of rhizobia, such as *Bradyrhizobium* species [12,13], *R. tropici* [14] and *R. loti* [15], incorporate into their nod factors common membrane fatty acids, such as $C_{18:1}$(vaccenic acid) or $C_{16:0}$ (the former has 18 carbon atoms and one double bond, the latter has 16 carbon atoms and is fully saturated). In these rhizobia, no host-specific nodulation gene products are involved in the biosynthesis of the acyl substituent, and the identity of the acyl substituent has little influence on nod factor activity on the respective host plants [16].

The recent studies demonstrating inter-species variability of the enzymatic activities of common nodulation gene products focused on the NodA and NodC proteins. Thus, genetic experiments showed that not all *nodA* genes are equivalent. Genetically engineered strains of *R. leguminosarum* bv. *viciae*, differing only in the source of their *nodA* gene, were tested for their ability to nodulate several

Figure 1



The major steps in formation of nitrogen-fixing nodules in *Rhizobium*–legume symbiosis. As shown, the nodulation genes of the bacterium are linked and carried on a 'Sym' plasmid.

natural hosts of *R. leguminosarum* bv. *viciae* [17]. The transconjugant containing the *nodA* gene from *Bradyrhizobium* was not able to induce nodule formation on the usual *R. leguminosarum* bv. *viciae* hosts. This phenotype was traced to the inability of the *Bradyrhizobium* NodA protein to incorporate the polyunsaturated fatty acid $C_{18:4}$, which is commonly found in *R. leguminosarum* bv. *viciae* nod factors.

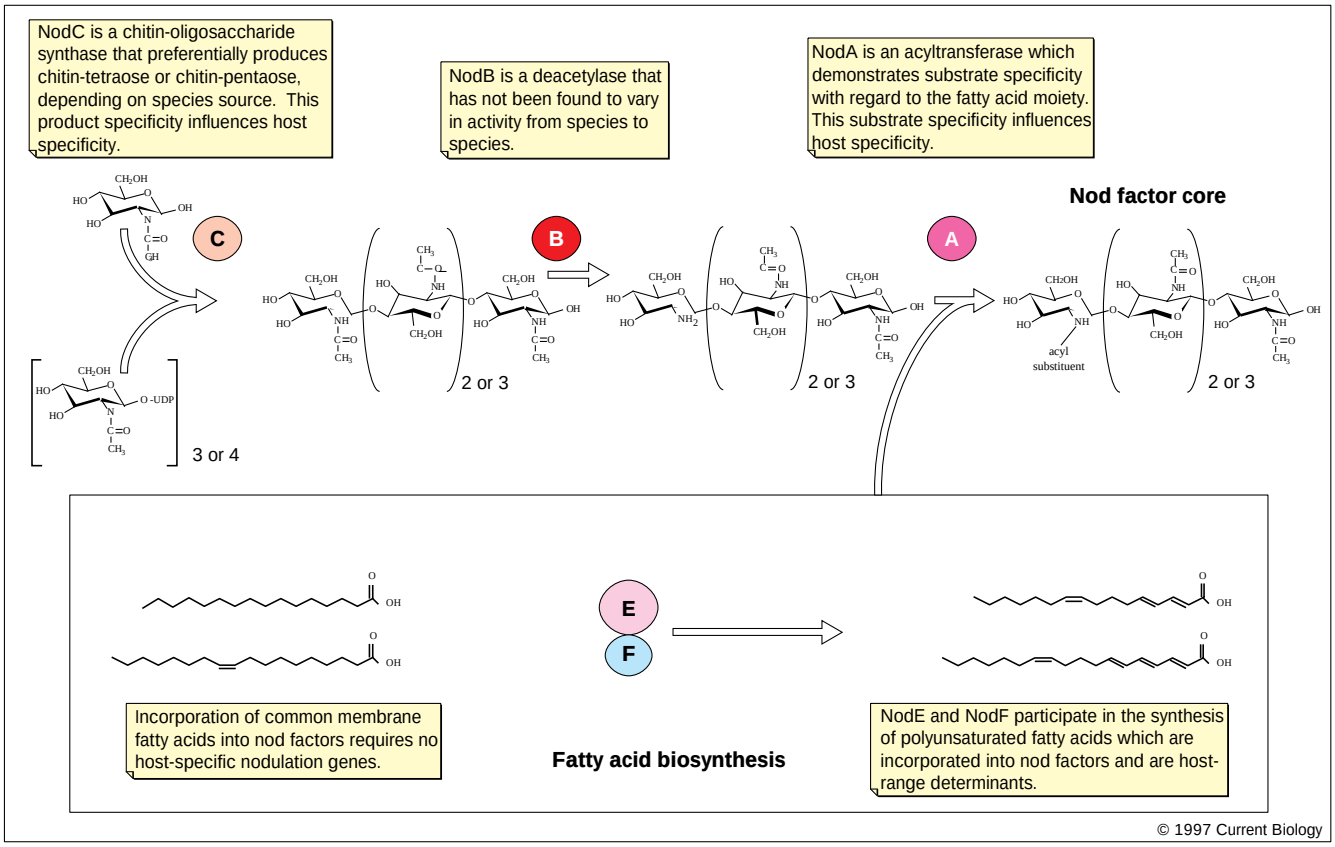
The NodA proteins from *R. tropici* and *R. meliloti* have similarly been found to have non-identical functions [18]. In these experiments, *nodA* function was supplied to a *nodA* mutant strain of *R. meliloti* by cloned *nodA* genes from *R. meliloti* or *R. tropici*. *R. meliloti nodA* conferred the ability to nodulate alfalfa with normal kinetics, whereas the transconjugant expressing *R. tropici nodA* nodulated alfalfa with severely delayed kinetics. Structural analysis of the nod factors produced by these two strains revealed that the nod factors differed in their acyl substituents. The strain expressing *R. meliloti nodA* produced nod factors with the polyunsaturated and ω-hydroxylated fatty acids characteristic of *R. meliloti* nod factors, whereas the nod factors from the transconjugant expressing *R. tropici nodA* contained only vaccenic acid. This shows that *R. tropici* NodA is unable to incorporate polyunsaturated and ω-hydroxylated fatty acids into nod factors. The ratio of chitin-tetraose to chitin-pentaose nod factors was unaffected by the source of NodA, so NodA appears not to have a role in determination of the length of the chitin backbone.

The *R. meliloti* and *R. leguminosarum* bv. *viciae* NodA proteins prefer to acylate chitin-oligosaccharides with polyunsaturated fatty acids determined by the host-specific *nodEF* genes. In the absence of the *nodEF*-determined polyunsaturated fatty acids, however, these NodA proteins will acylate the chitin-oligosaccharide with common membrane fatty acids. The preference of the *R. meliloti* and *R. leguminosarum* bv. *viciae* NodA proteins for *nodEF*-determined fatty acids, despite the large excess of common membrane fatty acids present in the cell, suggests that the selectivity of NodA is conferred by the donor of the acyl moiety. NodF is an acyl-carrier protein that is involved in the synthesis of the polyunsaturated fatty acids: an interaction between the NodA and NodF proteins may explain this selective fatty acid incorporation into nod factors.

Two recent studies [19,20] have examined the influence of NodC activity on nod factor backbone length and host range. Experiments with *R. tropici* transconjugants carrying subsets of the *R. meliloti* nodulation genes demonstrated the propensity of *R. meliloti* NodC to synthesize chitin-tetraose and *R. tropici* NodC to synthesize chitin-pentaose [20]. When NodC activity is isolated from the influence of other *nod* gene products, by expression of *nodC* in *Escherichia coli* or in *in vitro* experiments, the ratio of chitin-tetraose to chitin-pentaose produced is a characteristic of the individual NodC proteins [19]. The influence of other genes on chain length is suggested by the finding that the *R. loti* NodC protein can synthesize both chitin-tetraose and chitin-pentaose in *E. coli*, but only chitin-pentaose derivatives are found in wild-type *R. loti* cells. These studies demonstrate that the differences in nod factor backbone length between the various rhizobia species are largely dependent upon the NodC protein, but may also be influenced by additional rhizobial genes.

The recent experiments showing host-specific activities of NodA and NodC activities are not the first challenges to the notion that certain nodulation genes have common functions in the various rhizobial species. The *nodD* gene product has been known for several years to influence host range [21]. In the presence of plant-derived flavonoids,

Figure 2



Synthesis of the nod factor core by common nodulation gene products, NodA, NodB and NodC, and the host-specific nodulation gene products, NodE and NodF.

NodD activates the transcription of other *nod* genes. The responses of various NodD proteins were found to vary depending on the flavonoids produced by the plant, thus affecting host range of the rhizobia. Like the *nodA* and *nodC* genes, the homologous *nodD* genes were found to have analogous, but not identical, functions in symbiosis.

This inter-species variation in the enzymatic properties of common nodulation gene products is consistent with what we know about their molecular phylogeny. The phylogeny of the common nodulation genes mirrors the phylogeny of the host plants, rather than that of rhizobial species based on 16S RNA gene sequences [22]. This suggests that the inter-species variation in common nodulation gene function is driven by the interaction with the legume host. Thus, some distantly-related rhizobial species have similar host-ranges and NodA proteins with similar substrate specificities, whereas some other closely-related rhizobial species have different host-ranges and NodA proteins with different substrate specificities.

These findings taken as a whole suggest that the concept of 'common' nodulation genes having equivalent functions must be revised. A revised view of these genes should focus on what is 'common' or shared by the *nodD* and *nodABC* genes of different species. The shared features are their homologous sequences, analogous functions and presence in all rhizobial species studied so far. For other nodulation genes, such as *nodEF*, homologues with analogous functions do occur in multiple species, but they are not found in all rhizobia species and thus are not common to all rhizobia. Therefore, despite the contribution of some common nodulation genes to host-range determination, this modified view of common nodulation genes should continue to be useful in the study of *Rhizobium*–legume symbiosis.

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